Comparison of an inducible oxidative burst in free-living and symbiotic dinoflagellates reveals properties of the pseudopterosins

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Abstract

An oxidative burst in free-living and symbiotic dinoflagellates induced by physical stress is defined and characterized. The oxidative burst occurred within 1 min of physical injury caused by short pulses of low frequency sonic sound (20 kHz, 10 s pulses). The quantities of reactive oxygen species were measured using a spectrofluorometric assay and standardized to hydrogen peroxide. Using pharmacological probes, the oxidative burst was found to contain upwards of 95% hydrogen peroxide and was believed to be of enzymatic origin. Symbiotic dinoflagellates of the genus *Symbiodinium* sp. isolated from the gorgonian coral *Pseudopterogorgia elisabethae* produced a burst that was less than 33% of the magnitude of the oxidative burst in *Symbiodinium* sp. isolated from *Pseudopterogorgia americana* and less than 2% of the magnitude of the oxidative burst in the related free-living dinoflagellate, *Heterocapsa pygmaea*. We recently reported that *Symbiodinium* sp. from *Pseudopterogorgia elisabethae* contain high levels of the unique diterpene metabolites, the pseudopterosins, not found in the free-living *Heterocapsa pygmaea* and *Symbiodinium* sp. isolated from *Pseudopterogorgia americana*. Pseudopterosins completely blocked the inducible oxidative burst when applied exogenously to these two dinoflagellate species. Preliminary evidence is presented that raises the possibility that the mechanism could involve inhibition of G-protein activation among many possibilities. These data are the first description of an inducible oxidative burst in dinoflagellates. The significance of the comparative oxidative burst in free-living and symbiotic dinoflagellates is discussed and the potential natural role of the pseudopterosins is postulated.
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1. Introduction

The biological production of hydrogen peroxide and reactive oxygen species (ROS) as an immune response to pathogens has been well documented during phagocytosis in human neutrophils and mouse macrophages (Morel et al., 1991; Baldridge and Gerard, 1933; Pick et al., 1980; Dwyer et al., 1996) as well as a ubiquitous defensive response to injury and pathogenic invasion in higher plants (Bolwell et al., 1995; Bolwell and

Wojtaszek, 1997). In most of these cases the production of ROS occurs as a membrane NADP(H) oxidase mediated oxidative burst. The oxidative burst is an induced release of ROS such as superoxide (O^{2-}), hydroxyl radicals ($^{\cdot}OH$) or hydrogen peroxide (H_2O_2), which leads to acute cytotoxic effects to the pathogen or as signaling molecules for the induction of secondary defensive responses.

Hydrogen peroxide is an ROS that exists in seawater at concentration that vary 10-fold, with measurements ranging from 10^1–10^2 nM (Wong et al., 2003). It has been suggested that the main source of hydrogen peroxide in seawater is from algal photosynthesis and photochemical interactions, with secondary input from atmospheric deposition and localized release of pulses of H_2O_2 when some marine organisms experience biological stress (Wong et al., 2003; Palenik and Morel, 1988; Palenik et al., 1987). Thus far the biological production of ROS as an oxidative burst has been described in a limited number of marine species. Kupper et al. (2001) found an oxidative burst in the brown alga *Laminaria* in response to oligosaccharide elicitors and Ross and Jacobs (2003) found an oxidative burst induced by physical wounding in the green alga *Dasycladus vermicularis*. Collen and Davison (1999) also found that the brown alga *Fucus* spp. can produce increased amounts of ROS in response to physical stressors. There are a number of publications in the literature which measure elevated ROS levels due to high light irradiation and in response to environmental stressed conditions such as temperature variations, nutrient depletion and heavy metal contamination in phytoplankton. These include the raphidophyte flagellates *Heterosigma* (Twiner and Trick, 2000), *Olisthodiscus* (Kim et al., 1999b) and *Chattonella* (Oda et al., 1994; Kim et al., 2000) and the dinoflagellates *Cochlodinium* (Kim et al., 1999a) and *Symbiodinium* sp. (Lesser, 1996, 1997). In these cases the mechanisms of ROS production outside of photochemical reactions were not elucidated in detail or in the context of current signal transduction technology.

In the present study, we characterize an inducible oxidative burst in symbiotic dinoflagellates of the species *Symbiodinium* (Clade B1) isolated from the gorgonian coral *P. elisabethae* (PE) and the a close sister taxa dinoflagellate *Heterocapsa pygmaea* (a.k.a *Glenodinium* sp.; Loeblich et al., 1981; Santos et al., 2002) in response to physical injury. Mechanical injuries such as vigorous shaking (Legendre et al., 1993) and osmotic pressure (Yahraus et al., 1995) have been shown to induce an oxidative burst in cultured soybean cells. These studies convey the importance of inducible defense mechanisms in response to mechanical and physical stress as a generalized response to pathogen invasion (Yahraus et al., 1995). Sonic sound is an additional mechanical injury which can induce an oxidative burst and is a well studied abiotic elicitor of plant defense responses (Lin et al.,

2001). The sublethal effects of low intensity sonic sound causes increased membrane permeability, increased membrane ion fluxes, production of ROS and the increasing production and release of defensive secondary metabolites in plant cells (Lin et al., 2001; Wu and Lin, 2002). Due to the fact that sonic sound exposes the cells to various physical stressors, we restricted our study to this mode of action and we report here the kinetics and characterization of this oxidative burst in *Heterocapsa pygmaea* and PE *Symbiodinium* sp.

As part of this assessment of the inducible oxidative burst in *Symbiodinium* sp. isolated from PE, we test a hypothesis that this dinoflagellate cell has a resistance to injury relatable to high cellular concentrations of the diterpenoid marine natural products, the pseudopterosins present in these dinoflagellate cells (Mydlarz et al., 2003). The pseudopterosins are potent anti-inflammatory, analgesic and membrane stabilizing compounds with a unique mode of action (Look et al., 1986; Ettouati and Jacobs, 1987; Mayer et al., 1998; Moya and Jacobs, 2003). Using *H. pygamaea* as a dinoflagellate model, we studied the effects of pseudopterosin administration in reducing and preventing the oxidative burst.

2. Results and discussion

2.1. Induction and characterization of the oxidative burst due to physical injury in PE Symbiodinium sp. and H. pygmaea

PE *Symbiodinium* sp. and *H. pygmaea* cultures were subjected to a non-lethal dose of low frequency sonic sound (20 kHz) in 3 (10 s) pulses. The low intensity and short pulses of sonic sound used in this experiment did not cause cell lysis. At the 20 kHz frequency it takes several minutes of sonication in order to completely disrupt microalgal membranes (Branson, 1974). These sonic bursts cavitate the cells and thereby permeabilize the cell membrane causing a physical stress. In this study we first investigated the oxidative burst using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) visualized under epifluorescence microscopy. As can be seen in Fig. 1, normal control cells of *H. pygmae* and PE *Symbiodinium* sp. fluoresce red, while sonic sound produced green fluorescence which corresponds to the ROS produced from the oxidative burst. In order to quantify the amounts of ROS produced during an oxidative burst, a spectrofluorometric protocol was developed that quantified the oxidative burst in pmol H_2O_2/cell employing the same fluorescence probe.

Physically injured PE *Symbiodinium* sp. produced a small oxidative burst of 0.042 ± 0.0045 pmol H_2O_2/min/cell, while quite unexpectedly, the free-living model
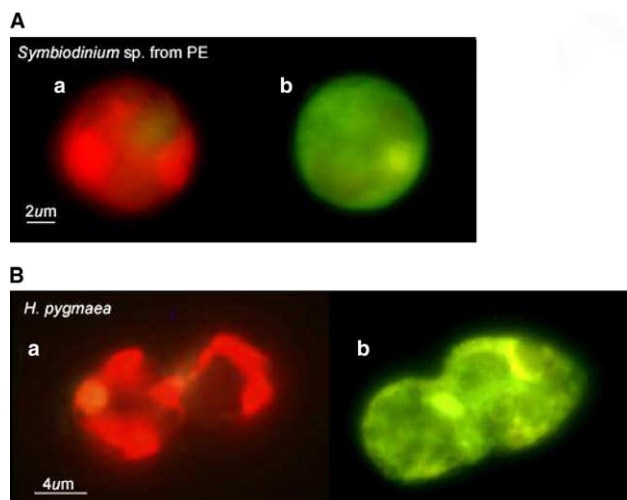
A

B

Fig. 1. (A) Epifluorescent micrograph of (a) uninjured *Symbiodnium* sp. from PE and (b) physically injured *Symbiodinium* sp. from PE. (B) Epifluorescent micrograph of (a) uninjured *H. pygmaea* and (b) physically injured *H. pygmaea*. Excitation 488 nm, emission 510 (longpath) under these conditions red indicates chlorophyll and green fluorescence indicates presence of ROS which reacts with DCFH-DA.

dinoflagellate, *H. pygmaea* produced an oxidative burst of 3.37 ± 0.26 pmol H_2O_2/min/cell, that was nearly 80 times greater than that of PE *Symbiodinium* sp. even though the two cells are similar in size and genetically related. In kinetic studies of the oxidative burst over time (Fig. 2), the peak of H_2O_2 released in both dinoflagellate species was reached within 1 min of the sonic sound pulses and the levels of H_2O_2 remained high for 10 additional minutes. While PE *Symbiodinium* sp. recovered from the burst after 20 min as levels of H_2O_2 approached zero, *H. pygamaea* cells did not recover and the levels of H_2O_2 showed a gradual decrease from the maxima at 3.37 pmol/cell to approximately 2.21 pmol/cell for the remainder of the period tested (40 min). The rate of H_2O_2 decrease of *H. pygmaea* cells was calculated as 0.031 pmol/min/cell and at this rate it would take a total of 110 min for the cells to return to baseline H_2O_2 levels. This slow reduction of H_2O_2 levels is probably due to combined slowing of the production rate and enzymatic detoxification.

To begin to elucidate the enzymatic origin and the chemical composition of the oxidative burst in *H. pygmaea* and PE *Symbiodinium* sp. in response to physical injury, the cells were incubated with diphenylene iodonium chloride (DPI) and catalase. DPI is a irreversible inhibitor of NAD(P)H oxidase and a weak inhibitor of peroxidases at high concentrations (greater than 100 μM) (Bolwell et al., 1998). In the current study 50 μM of DPI inhibited the oxidative burst by 100% ($n = 3$) in PE *Symbiodinium* sp. cells and by $95 \pm 2.2\%$ ($n = 3$) in *H. pygmaea* cells. The complete inhibition of the sonic sound elicited oxidative burst by DPI is consistent with presence of superoxide-generating NAD(P)H oxidases

which are constitutive enzymes in the oxidative bursts of mammalian neutrophils and higher plants (Bolwell et al., 1995; Kupper et al., 2001; Dwyer et al., 1996). In addition, DPI has been shown to be an inhibitor of H_2O_2 production by peroxidases in French bean cells (Bolwell et al., 1998). Complete inhibition of H_2O_2 production in French bean cells was not achieved using DPI even at concentrations above 100 μM (Bolwell et al., 1998) further cloning experiments positively identified the peroxidase responsible for the oxidative burst in French bean cells (Blee et al., 2001). While additional future experiments may identify the putative mechanism behind the oxidative burst in *Symbiodinium* sp. and *H. pygmaea*, production of ROS due to sonic injury in the present experiments are likely attributed to an enzymatic system either NAD(P)H oxidase or peroxidase in origin.

Catalase the enzyme which initiates the decomposition of hydrogen peroxide was used to determine the chemical species present in the oxidative burst. Catalase has a high selectivity for H_2O_2 and can rapidly convert the toxic molecules to water and oxygen (Kirkman and Gaetani, 1984) and follows an exponential rate law rather than Michaelis-Menton kinetics (Mueller et al., 1997). In many systems, such as human erythrocytes and rat cardiac metabolism, catalase is the predominant H_2O_2 removing enzyme (Mueller et al., 1997; Thayer, 1986). In PE *Symbiodinium* sp. 50 U/ml of catalase reduced the fluorescence emission to basal levels 5 min post sonic injury (Table 1). This may indicate that the oxidative burst in PE *Symbiodinium* sp. was composed of nearly 100% hydrogen peroxide. In *H. pygmaea* a range of 50–150 U/ml was used to inhibit the oxidative burst in a 5 min incubation post injury. The anti-oxidant effects of catalase were dose-dependant and the rate of hydrogen peroxide decomposition increased with increasing catalase concentrations (Table 1). A steady decrease of fluorescence emissions was observed and the highest dose of 150 U/ml decreased the H_2O_2 levels to near basal levels. In these experiments complete reduction of H_2O_2 never reached basal levels or below, this may indicate that the oxidative burst in *H. pygmaea* is composed mainly of H_2O_2, but may also have a fraction of other free radicals that react with the fluorescent probe. The high levels of catalase necessary to inhibit the oxidative burst in *H. pygmaea* also support the observation that this free-living dinoflagellate is releasing a higher concentration of ROS than PE *Symbiodinium* sp. The oxygen radical scavenger, ascorbic acid, stoichiometrically scavenged all the sonic sound elicited ROS when added in excess (200 μM) within 1–3 min.

2.2. Oxidative burst in related symbiotic dinoflagellates

We compared the oxidative burst due to sonic injury in *Symbiodinium* sp. freshly isolated from PE, from a
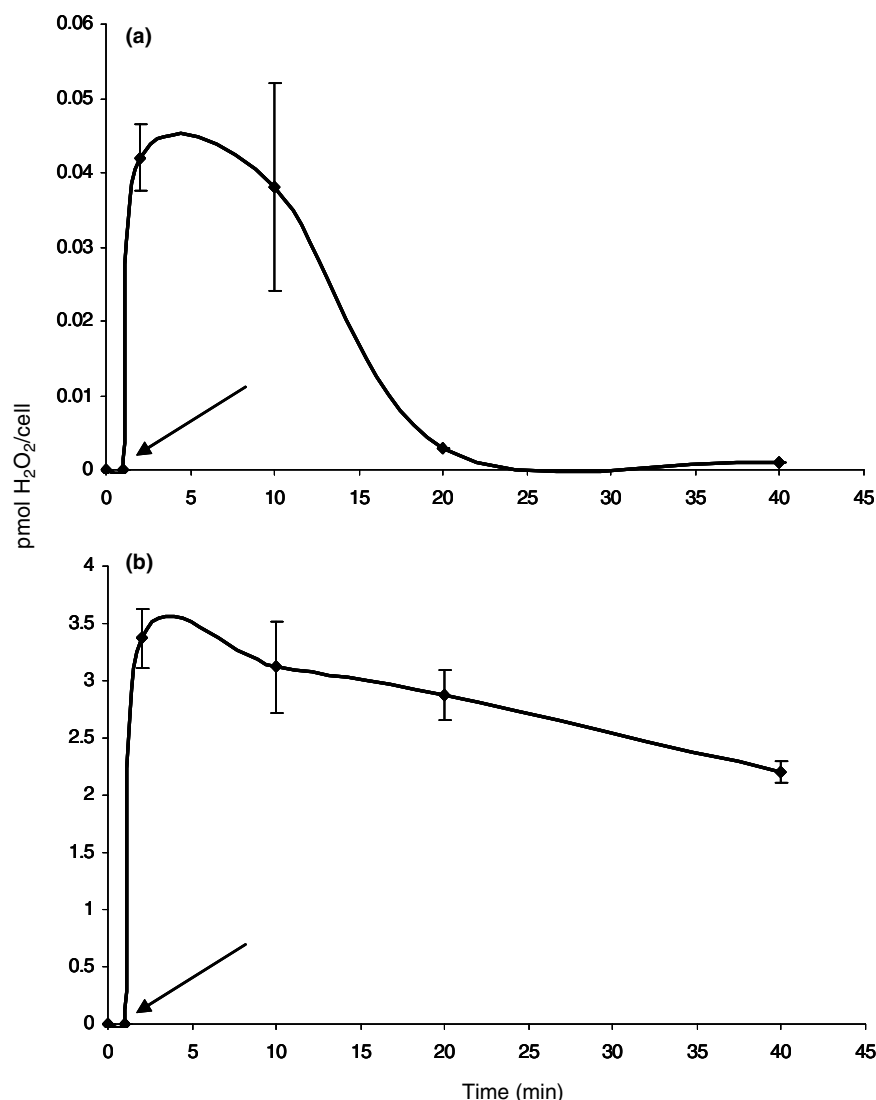
Fig. 2. Kinetics of the oxidative burst caused by sonic sound in (a) PE *Symbiodinium* and (b) *H. pygmaea*. ($n = 5$) Arrows indicate point of injury.

Table 1
Effects of catalase on PE *Symbiodinium* sp. and *H. pygmaea*

Treatment	PE *Symbiodinium* sp. ($\mu M \ H_2O_2$)	Rate of H_2O_2 degradation by catalase ($\mu M/min$)	*H. pygmaea* ($\mu M \ H_2O_2$)	Rate of H_2O_2 degradation by catalase ($\mu M/min$)
Basal H_2O_2 level	10 ± 0.3	N/A	53 ± 5.0	N/A
Injured	16 ± 0.4	N/A	280 ± 1.0	N/A
Injured + 50 U catalase	10 ± 0.1	1.2	143 ± 8.0	27.4
Injured + 100 U catalase	–	–	101 ± 10.0	35.8
Injured + 150 U catalase	–	–	73 ± 3.0	41.4

Data represents μM of H_2O_2 produced in 250,000 cells/ml, incubated for 5 min with catalase.

cultured strain of *Symbiodinium* sp. and *Symbiodinium* sp. freshly isolated from *P. americana* (PA) (Fig. 3). The cultured *Symbiodinium* sp. produced an oxidative burst almost twice the magnitude of PE *Symbiodinium* sp. and PA *Symbiodinium* sp. produced a burst three times greater than that of PE *Symbiodinium* sp. The relatively muted oxidative burst of this select group of symbiotic dinoflagellates was on average 50 fold less than the burst in the free-living *H. pygmaea* (3.37 pmol H_2O_2/min/cell vs. a mean of 0.074 pmol H_2O_2/min/cell). This large difference in the magnitude of the oxidative bursts between symbiotic and free-living dinoflagellates represents an unexpected phenomena which may prove beneficial to the symbionts and could be related to the specific symbioses that these dinoflagellates have established with invertebrate hosts. Excess H_2O_2 can cause damage to
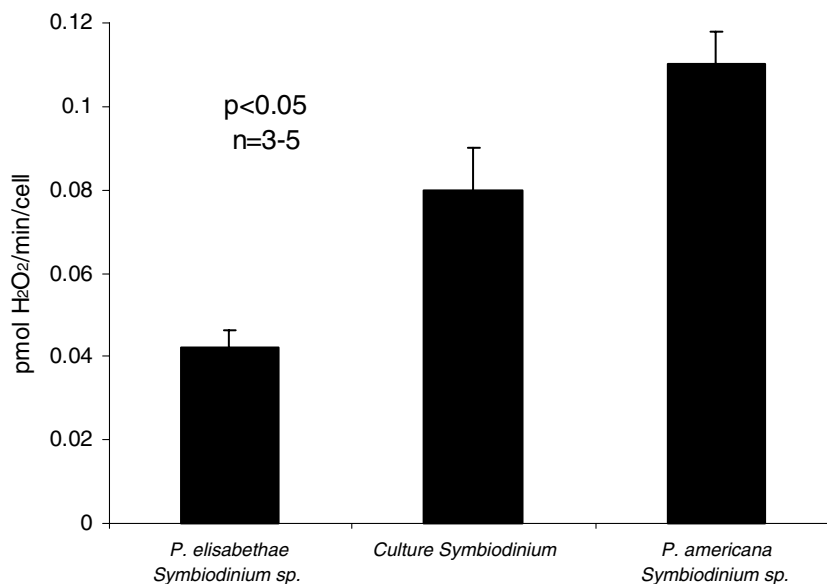
Fig. 3. Oxidative burst in *Symbiodinium* sp. Clade B1, from culture and freshly isolated from *P. elisabethae* and *P. americana* ($n = 5$). Experiments were carried out with individual coral colonies from multiple collections (see materials and methods for more details). Statistically significant differences between mean values $p < 0.05$ unpaired Student's t-test.

photosystem II at the D1 dimer protein within the symbiotic dinoflagellate (Richter et al., 1990). It can also diffuse into the host tissues where similar interference with membrane functions might occur (Asada and Takahashi, 1987). This has been reported to lead to decreased photosynthesis and diminished translocation of photosynthate and may cause exocytosis of the symbionts from the host cell (Lesser, 1997). These published results and the recent measurements reported here, raise the possibility that in addition to known anti-oxidant enzymes present in these cells, there may be distinctive host signals or natural products that are produced that down regulate or mitigate the synthesis of oxygen radicals.

The average lipid fraction of PE *Symbiodinium* sp. contains approximately 15% pseudopterosins which are potent anti-inflammatory and membrane stabilizing compounds, (Mydlarz et al., 2003). The PA *Symbiodinium* sp and the cultured *Symbiodinium* sp. do not contain these compounds. This fact, along with the observation in this study that PE *Symbiodinium* sp. exhibited a markedly greater resistance to sonic injury when compared with PA *Symbiodinium* sp. and *Symbiodinium* cultures raised the possibility that the unique lipid signature of PE *Symbiodinium* sp. may be contributing to a natural role in protecting against a damaging oxidative burst. Even though there are environmental, physiological and morphological differences between these symbiont cells and coral hosts, all the *Symbiodinium* species used in this study were identified as Clade B1 sub-type by the ITS-rDNA region genotyping technique (LaJeunesse, 2001, 2002). More specifically, PE and PA *Symbiodinium* sp. have the same number of

base pairs of the cp 23SrDNA domain V allele (184 bp) (Santos et al., 2002) thus the two symbionts are phylogenetically similar.

Heterocapsa pygmaea is a common free-living dinoflagellate that has been used extensively as a model for physiological comparisons with symbiotic dinoflagellates due to their similar sizes and the ease of culturing (Santos et al., 2003; Govind et al., 1990; Iglesia-Prieta et al., 1993; Boczar and Prezelin, 1986). *Heterocapsa* as a genus is representative of many dinoflagellate genera as they are planktonic and pandemic (Watson and Loeblich, 1983) they are environmentally important because they are bloom-forming and they have been extensively studied for their photophysiology (Johnsen et al., 1994, 1997; Nelson and Prézelin, 1990; Triplett et al., 1993). Since they produced a large oxidative burst in our studies, lack pseudopterosin chemistry and are genetically related to *Symbiodinium*, *H. pygmaea* was investigated to test the effects of pseudopterosin addition on the physically induced oxidative burst.

2.3. Inhibition of the oxidative burst elicited by sonic sound in H. pygmaea by the pseudopterosins

The effects of the pseudopterosins on the oxidative burst caused by injury in *H. pygmaea* were examined using a formulation of pseusopterosins A, B, C and D in their natural constitution (ratio of 15% PsA, 24% PsB, 38% PsC and 22% PsD). Pseudopterosin A, B, C and D (**1**–**4**) have the same tricyclic diterpene backbone, but differ in acetylation of the glycosidic moiety (Fig. 4c)

(Look et al., 1986). *H. pygamea* cells were pre-treated with various concentrations of the pseudopterosin **1–4** for 1 h prior to sonic injury. As can be seen in Fig. 4a, the pseudopterosins **1–4** inhibited the oxidative burst in a concentration dependent manner (approximate IC_{50} of 8 μM). The dose–response curve followed pseudo-first order kinetics (c.v. 0.95), with the reaction rate of H_2O_2 production declining exponentially with increasing pseudopterosin **1–4** concentrations. A semi-log plot (Fig. 4b) shows that the rate of inhibition rises sharply between 7 and 10 μM, with near saturation at concentrations greater that 10 μM. This sharp incline in the inhibition curve may be caused by complex pseudopterosin receptor interactions, where the effect of one pseudopterosin glycoside potentiates, augments or alters the effect of the other pseudopterosins in the mixture. This cooperative interaction would cause a large positive increase in the physiological effects of the compounds. Another reason that the percent inhibition curve has a

steep incline at 7 μM may be due to the fact that all these experiments were conducted with a homogeneous population of *H. pygmaea* that were genetically identical. Likewise, since sonic sound intensely agitates the cells and permeabilizes cell membranes, the pseudopterosins **1–4** may exhibit general cell membrane stabilization properties in addition to inhibition of the oxidative burst. Stabilizing effects of the pseudopterosins have been observed in fertilized sea urchin embryos (Ettouati and Jacobs, 1987) and in calcium ionophore stimulated neutrophils (Mayer et al., 1998).

Despite many mechanistic and pharmacological studies of the pseudopterosins, the mechanism of action remains undefined. Mayer et al. (1998) found that the pseudopterosins do not inhibit phospholipase A_2 or phospholipase C, they do not block eicosanoid biosynthesis from arachidonic acid and the compounds do not inhibit the lipoxygenase or cycloxygenase enzymes. Recent evidence from our laboratory (Moya and Ja-
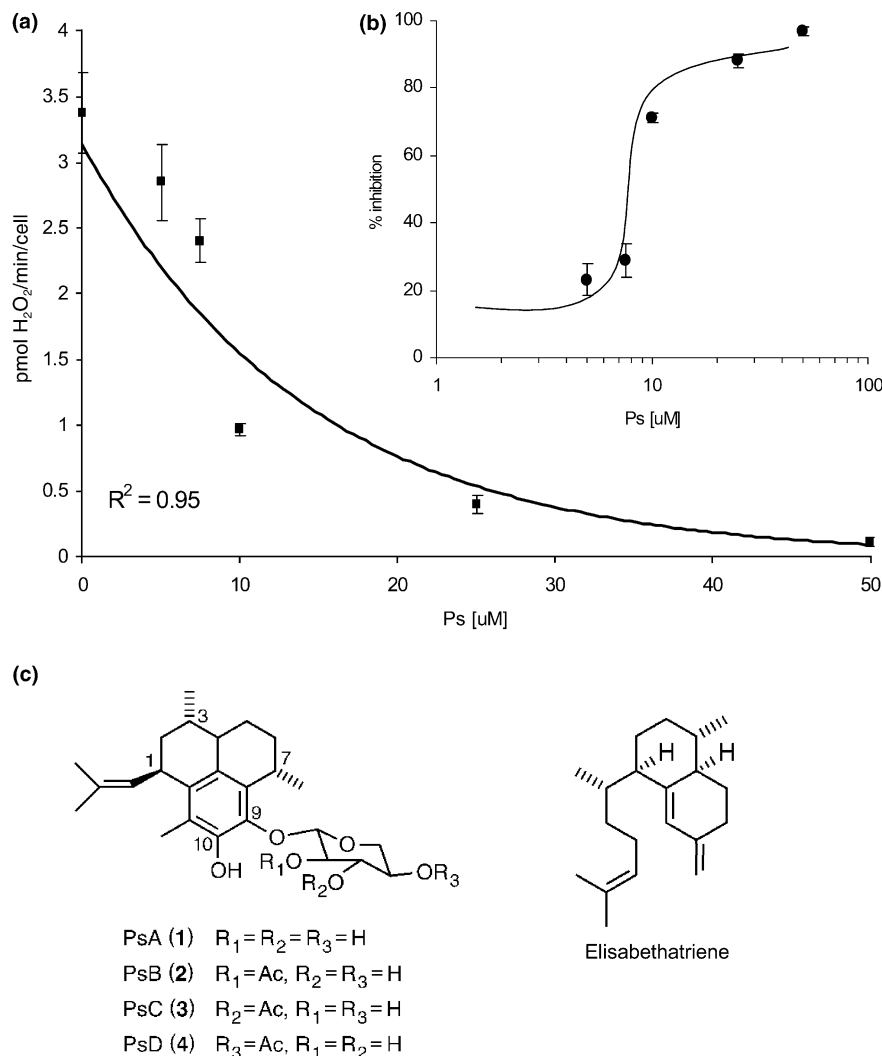

Fig. 4. (a) Inhibition of oxidative burst in *H. pygmaea* by the pseudopterosins. (b) Semi-log dose–response curve of pseudopterosins inhibition of oxidative burst. (c) Structures of the pseudopterosins (**1–4**) and elisabethatriene (**5**); used in this study.
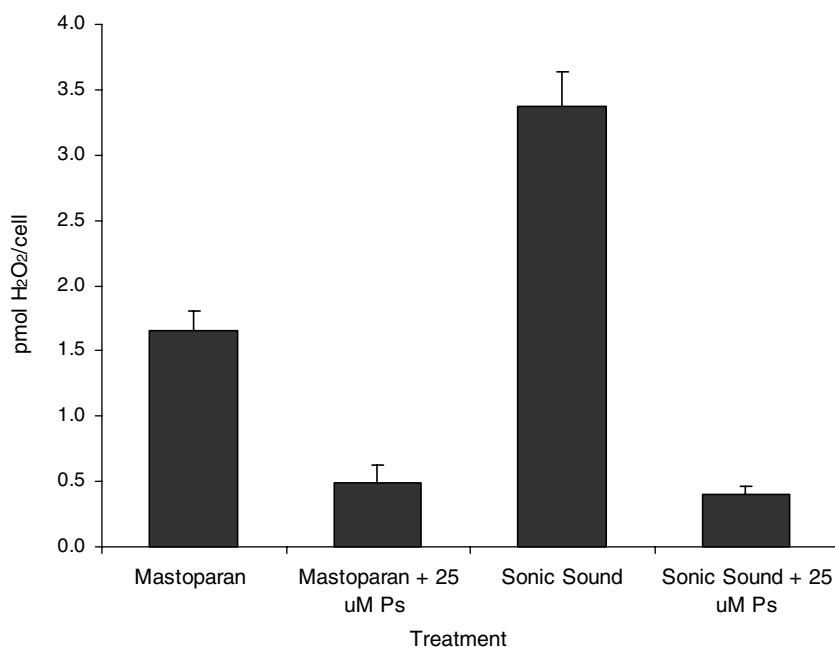
Fig. 5. Comparison of the G-protein elicitor mastoparan and sonic sound on the induction of the oxidative burst in *H. pygmaea*.

cobs, 2003) suggests that the pseudopterosins are acting on a pertussis sensitive G-protein and may have a novel mechanism of action. In order to further define this pathway and the inhibitory effects of the pseudopteroins on the oxidative burst in *H. pygmaea* we conducted experiments with pharmacological elicitors of the oxidative burst independent from mechanical injury (Fig. 5). Mastoparan, a G-protein activator isolated from wasp venom induced an oxidative burst in *H. pygmaea* in absence of sonic sound cavitations. The magnitude of the oxidative burst due to 10 μM mastoparan was 1.65 ± 0.16 pmol H_2O_2/cell and occurred after 12 min of treatment with the toxin. This response was approximately half the magnitude of the oxidative burst due to low frequency sonic sound cavitations. Addition of 25 μM of the pseudopterosins 1 h prior to treatment of mastoparan inhibited the oxidative burst by 71% providing initial evidence that the pseudopterosins may modulate G-protein activation. Further experiments are underway to elucidate the definitive target sites of mastoparan in causing an oxidative burst and the site at which the pseudopterosins may act.

In addition to testing the inhibitory effects of the pseudopterosins **1–4** we administered 25 μM of elisabethatrienes to *H. pygmaea* cells (Fig. 4c). This bicyclic

hydrocarbon which is first committed intermediate of pseudopterosin biosynthesis (Coleman and Kerr, 2000) had no effect on the sonic sound elicited oxidative burst.

In other experiments, we compared the rate of inhibition in H_2O_2 production following treatment of PA *Symbiodinium* sp. and *H. pygmaea* with 25 μM of pseudopterosins. This concentration inhibited the oxidative burst by 80% in PA *Symbiodinium* sp ($n = 3$, $p < 0.01$) and by 86% in *H. pygmaea*, respectively (Table 2). The rate of H_2O_2 production was reduced from 0.1 pmol/min/cell to 0.02 pmol/min/cell in PA *Symbiodinium* sp. In contrast H_2O_2 production in *H. pygmaea* was reduced from 3.4 pmol/min/cell to 0.5 pmol/min/cell. Thus the absolute reduction in H_2O_2 was 36 times greater in *H. pygmaea* using the same concentration of pseudopterosins, mitigating against the view that a direct anti-oxidant/scavenging effect of the pseudoterosins is a primary mechanism.

To further evaluate the direct oxygen scavenging properties of the pseudopterosins an assay using a cell free system of water and H_2O_2 was developed and the direct antioxidant effects of the pseudopterosins were compared to the lipophilic scavenger, α-tocopherol. 5, 25 and 50 μM of each compound was added to a synthetic system of hydrogen peroxide and water and the

Table 2
Comparison of inhibitory effects of the pseudopterosins on PA *Symbiodinium* sp. and *H. pygmaea*

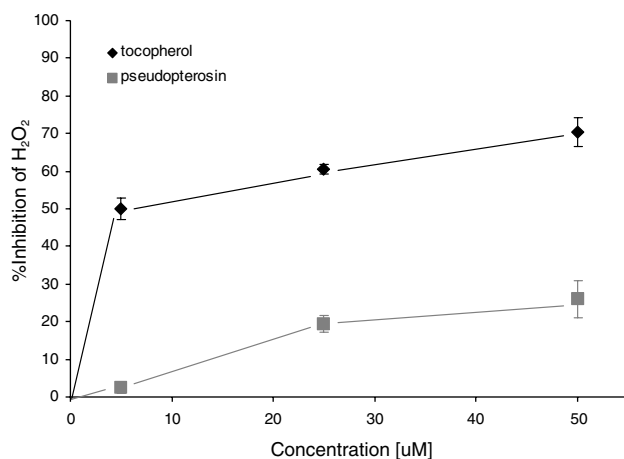| Treatment	PA *Symbiodinium* sp.	*H. pygmaea*
Sonic sound	0.10 ± 0.008 pmol/min/cell	3.37 ± 0.26 pmol/min/cell
Sonic sound + 25 μM pseudopterosins	0.021 ± 0.008 pmol/min/cell	0.50 ± 0.008 pmol/min/cell
% Inhibition	80%	86%
Absolute change in H_2O_2 production rate	0.079 pmol/min/cell	2.87 pmol/min/cell

Fig. 6. Anti-oxidant effects of the pseudopterosins and the known anti-oxidant tocopherol in synthetic system of H_2O_2 and water.

scavenging effects were measured by a decrease in fluorescence (Fig. 6). At 50 μM the lipophilic scavenger α-tocopherol scavenged 70% of the hydrogen peroxide available while at 50 μM the pseudopterosins scavenged a maximum of 26% of the hydrogen peroxide available. Thus the oxygen scavenging effects of the pseudopterosins appear to contribute minimally to the pharmacological effects of these compounds i.e. the inhibitory concentration at 50% (IC_{50}) for the pseudopterosins is only 8 μM in the presence of *H. pygmaea* cells.

2.4. Concluding remarks

The present study documents the oxidative burst that occurs under physical stress conditions in dinoflagellates, a taxonomic group of marine phytoplankton, well known for the symbiotic relationships they form with a number of marine invertebrates and, in its free-living form, for the formation of harmful algal blooms (e.g. red tides). It is the first characterization of an enzymatic oxidative burst in the genera of *Symbiodinium* sp. and *Heterocapsa*. We found that symbiotic dinoflagellates have a muted oxidative burst when compared to free-living *H. pygmaea* stressed under the same conditions. Particularly, the insensitivity to sonic membrane disruptions in PE *Symbiodinium* sp. may be in part be due to the presence of pseudopterosins that are concentrated within the cell (Mydlarz et al., 2003). *H. pygmaea* and PA *Symbiodinium* are both naturally devoid of pseudopterosins, however, exogenously added pseudopterosins to *H. pygmeae* and PA *Symbiodinium* provided the cells with similar protection and minimal response to physical injury. Therefore, we suggest a possible endogenous protective role for the pseudopterosins in the *P. elisabethae* coral–algal complex that may be ecologically significant to optimization of the symbiont lifestyle.

While the exact mechanism by which the pseudopterosins inhibit the oxidative burst remains unknown, the experiments reported here offer some preliminary insight into the effects of the pseudopterosins on the oxidative burst signal transduction cascade. The pseudopterosins are able to inhibit mastoparan G-protein agonist activities. In addition, the pseudopterosins may act as membrane stabilizers and we are not able to detect significant anti-oxidant properties for these molecules.

Our results suggest that enzymatically induced (biological) production of ROS in addition to photochemical production can be a significant source of oxygen radicals in the marine environment and in coral-symbiont relationships. The production of ROS as a result of an oxidative burst are known to have many functions in cell signaling and can alter protein and gene expression in metabolic pathways and lipid signals (Hensley et al., 2000). In dinoflagellates, several genes are expressed when the cells were under oxidative stress. In *Pyrocystis lunula* (Okamoto and Hastings, 2003) high ROS conditions caused the induction of genes involved in protein phosphorylation, signal transduction, photosynthesis and carbon metabolism, and the production of proteins for defense and detoxification of ROS molecules. More specifically, ROS have been implicated as signaling molecules in the pathways involved in cell growth, differentiation and apoptosis (Okamoto and Hastings, 2003). In the fresh water dinoflagellate, *Peridinium gatunense*, the production of H_2O_2 has been implicated in programmed cell death (Vardi et al., 1999) during dense bloom conditions that result in inorganic carbon depletion. In these studies the addition of catalase decomposed the H_2O_2 and apoptosis was prevented. While these published results do not identify the source of H_2O_2 production, clearly an enzymatic oxidative burst combined with non-enzymatic ROS sources together have an important role in algal bloom dynamics, especially when rapidly dividing cells accumulate and inorganic nutrients decline causing physiological stress on the algal population. On a physiological level, an oxidative burst can signal lipid peroxidation and formation of highly unsaturated fatty acids. These conjugated fatty acids are extremely reactive and are well known precursors to important cell signaling molecules (Hamberg, 1992). These lipid signals can also act as chemotactic factors and cause cell aggregation (De Petrocellis and DiMarzo, 1994).

Lastly, from an evolutionary prospective, the presence of the oxidative burst in unicellular symbiotic and free-living dinoflagellates may indicate a conservation of these pathways as defense mechanisms in macroalgae, higher plants and even mammalian immune systems. In recent years, the paramount importance of ROS in disease processes has been recognized. For instance, redox-sensitive genes, similar to those identified in *Pyrocystis lunula* by Okamoto and Hastings (2003) have been iden-

tified in protein kinase cascades which induce inflammatory gene expression in mammalian cells (Hensley et al., 2000). Perhaps the detection and detailed analysis of an enzymatic oxidative burst in ancient, unicellular organisms such as dinoflagellates may have a widespread significance in the understanding of immune responses which contribute to chronic and degenerative diseases (Hensley et al., 2000). Thus ultimately there may prove to be a direct relationship between the effects of pseudoptersoins on oxidative stress and their potent pharmacological activities.

3. Experimental

3.1. General experimental procedures

2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA or H_2DCFDA) was purchased from Molecular Probes, Eugene, OR. Diphenylene iodium chloride (DPI), Ascorbic acid, esterase (41 U/mg), catalase (3250 U/mg) were purchased from Sigma. Perkin–Elmer LS 50B Luminescence Spectrophotometer was used for fluorescence readings and HPLC purifications were performed using a Hitachi L-6200A Intelligent Pump connected to a L-4200 UV–Vis detector with Varian Chrompack analytical silica columns (20 cm, 4 mm diameter).

3.2. Algal cells

Cultures of *H. pygmaea* a.k.a *Glenodinium* sp. (Strain CCMP 1132) were generously provided by Dr. Barbara Prezelin and maintained at 17 °C in L1 media without Silica. Cells were harvested for experiments during stationary growth phase. *Symbiodinium* sp. culture 146 originally isolated from *Oculina* was generously provided by Dr. Todd LaJeunesse and maintained at 25 °C in f/2 media. Coral specimens were generously provided by Dr. Russell Kerr and Dr. Lory Santiago-Vasquez. *P. elisabethae* was collected in May of 2003 and January of 2004 at Sweetings Cay in the Bahamas at a depth of 10 m. *P. americana* was collected in Long Key at 5 m depth. Live coral were homogenized in a blender with 0.22 μm filtered seawater and 10 mM EDTA, and filtered through 4 layers of cheesecloth. Algal symbionts were pelleted out by centrifugation at $250 \times g$ and subsequently washed 10 times with 40 ml clean filtered seawater and pelleted by centrifugation at $750 \times g$. *Symbiodinium* sp. cells were further purified on Percoll® step gradient of 20%, 40%, and 80% two or more times until <1% impurities were seen using light microscopy. Cell viability was confirmed using trypan blue incorporation. Cells were maintained at 25 °C in filtered seawater.

3.3. Injury and drug treatment of cells

Cells at 5×10^5 cells/ml were subjected to three 10 s pulses of 20 kHz sonic sound on a Fisherbrand sonic dismembranor 200. Cell viability and shape post injury was measured using trypan blue incorporation under light microscopy. For chemically injured cells drugs were added in minimal DMSO or EtOH. Vehicle controls were used all experiments. A release of ROS from *H. pygmaea* but not from any *Symbiodinium* species was observed in response to vigorous shaking.

3.4. Oxidative burst assay

One ml of live cells pre and post injury was added to 970 μl of sea water, 20 μl esterase (1 mg/ml, U) and 10 μl of 10 mM DCFH-DA (0.5 mM final concentration) and stirred. The reaction mixture was read on a Perkin–Elmer LS 50B Luminescence Spectrophotometer, excitation 488 nm and emission 525 nm. Results were recorded after 3 min. The relative DCFH-DA fluorescence of the oxidative burst was calibrated to a standard curve of hydrogen peroxide prepared with 1.7 ml seawater, 20 μl esterase and 0.5 mM DCFH-DA and various concentrations of 30% H_2O_2 prepared as a 5 mM stock in DI H_2O. All experiments were repeated 3–10 times, data presented consists of mean and standard error of independent experiments.

3.5. Isolation of pseudopterosins

P. elisabethae was extracted using HPLC grade $CHCl_3$ and EtOAc as previously described (Mydlarz et al., 2003). Briefly, crude extracts were partitioned between MeOH/H_2O (9:1) and hexanes, followed by partitioning between MeOH/H_2O (1:1) and $CHCl_3$. Pseudopterosins A through D (1–4) were purified on a Hitachi L-6200A intelligent pump connected to a Hitachi L-4200 UV–Vis detector with Varian Chrompack silica column using a hexanes/EtOAc gradient (60:40 to 100% EtOAc in 40 min; flow rate 1 ml/min) with UV detection at 283 nm. Peak areas corresponding to the pseudopterosins were used to quantify amounts from standard curve equations previously obtained.

species identification and cultures of *Symbiodinium* sp. (culture 146). The authors would also like to thank two anonymous reviewers whose comments helped to greatly improve this paper. The authors are especially grateful to Frithjof Kupper for helpful discussions and insights while visiting our laboratory.

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